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## 14. ABSTRACT

The high level exposure of our troops to ultraviolet (UV) radiation increases their risk of melanoma. Notably, melanoma is the second most common cancer in males in the US Navy. Melanoma is an extremely aggressive disorder. Once invasive, it rapidly metastasizes to distant organs, the response to chemotherapeutic regimens is poor, and the five year survival rate is low. Thus, new chemotherapeutic strategies must be devised to reduce the numbers of our military, veterans and members of the general public succumbing to melanoma. We aim to understand resistance mechanisms in melanoma in order to provide the basis for improved targeted therapeutic strategies.

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**Introduction**

The high level of exposure of our troops to UV radiation increases their risk of melanoma, the deadliest form of skin cancer. Melanoma is characterized by frequent V600E mutations in the serine-threonine kinase, B-RAF. In phase 1-3 trials, a B-RAF inhibitor, PLX4032/vemurafenib, elicited objective initial responses in approximately 50% of melanoma patients harboring B-RAF mutations [1]. Response correlates closely with >80% inhibition of ERK1/2 activity in patients [2]. However, the response was heterogeneous. The mechanisms underlying intrinsic resistance to B-RAF inhibitors are unknown and must be elucidated to optimize future clinical trials. This application focuses on our recent finding that a stemness-related factor, FOXD3 is up-regulated following inhibition of mutant B-RAF signaling in melanoma cells. We hypothesize that up-regulation of a stemness transcription factor, FOXD3, is an adaptive response that promotes resistance to PLX4032-induced apoptosis in mutant B-RAF melanoma cells.

**Body**

The objective of this application is to determine mechanisms underlying resistance to PLX4032 and its tool compound, PLX4720. We proposed to: 1) determine the role of FOXD3 in resistance to PLX4032/4720; 2) define the role of FOXD3 in resistance to apoptosis and stem cell-like properties of melanoma cells; and 3) identify mechanisms regulating FOXD3 in mutant B-RAF melanomas.

Task 1: To determine the role of FOXD3 in resistance to PLX4032/4720

i) Obtain local IACUC and DOD USAMRMC Office of Research Protection approval for the use of animals.

Protocol CA100311 entitled, "Signal Transduction in Melanoma," IACUC protocol number 853A was approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of mice. This protocol was also approved by the Thomas Jefferson University IACUC.

ii) Knockdown FOXD3 in mutant B-RAF melanoma cells in xenografts and determine effects on PLX4720-induced tumor regression.

Since FOXD3 may elicit effects on the tumor bulk and/or a small population of drug tolerant cells, we proposed to test the effects of FOXD3 depletion on tumor regression with PLX4720. We initially tested the duration of effects of short-interfering RNA (siRNAs) to deplete FOXD3 levels since we have utilized siRNAs in the preliminary data associated with the application. We showed that siRNA knockdown of FOXD3 lasted between 9 and 12 days. Since typical tumor regression experiments last for 4 weeks, this approach was deemed not to be sufficient for our in vivo experiments. Next, we set up lentiviral-based short-hairpin RNA (shRNAs) systems to persistently knockdown FOXD3. This would overcome the short-term effects of siRNAs. We have currently engineered these constructs and identified multiple sequences to knockdown FOXD3. Since the use of shRNAs has concerns about off-target action, we are taking the addition step of generating erase-and-replace system, in which we can re-express FOXD3.

The second aspect of this work is to detect and quantify the response to RAF inhibitor. To this end, we have generate luciferase-expressing melanoma cells to facilitate longitudinal live whole animal imaging in a CALIPER-IVIS system. We have generated these cells and have established conditions for the inhibition of tumor growth in vivo with PLX4720.

iii) Knockdown FOXD3 in mutant B-RAF melanoma cells in xenografts and determine effects on tumor re-growth following PLX4720 withdrawal.

These studies aim to analyze the effect of FOXD3 depletion on re-growth following PLX4720 treatment cessation. These experiments are subsequent to the analysis in SubAim ii.

iv) Determine the association between FOXD3 up-regulation and relapse in clinical samples from the phase 2/3 PLX4032 trial.

These experiments aimed to test our hypothesis in samples from patients enrolled in the on-going PLX4032 trial. We are still in the process of obtaining samples from Dr. Jeff Sosman (Vanderbilt University).

Task 2. Define the role of FOXD3 in protection from apoptosis and stem cell-like properties of melanoma cells

i) Site-directed mutagenesis to make FOXD3 mutants.

To understand how FOXD3 acts, we proposed to define the domains required for protection from PLX4720-induced apoptosis in mutant B-RAF melanoma cells. We performed site-direct mutagenesis to generate the following mutants that were proposed in the application: 1) N187A/H191A mutations of conserved residues within the Forkhead domain that are required for the DNA binding activity of Forkhead proteins [3]; 2) serine 46 to alanine mutant (S46A) based on our proteomic data identifying serine 46 phosphorylation on FOXD3; 3) F378E mutant that mediates FOXD3 interaction with the Groucho proteins, which is critical for transcriptional repression [4]. For each of these FOXD3 mutants, we created expression systems in mutant B-RAF melanoma cells, as described [5] and performed erase-and-replace assays in which endogenous FOXD3 was depleted with the #18 FOXD3 3'UTR-targeting siRNA. Re-expression of wild-type FOXD3 served as a control.

ii) Express FOXD3 mutants and determine effects on resistance to PLX4032/4720.

The N187A/H191A, S46A, F378E mutants in task i) were utilized in annexin V staining assays to determine the ability of FOXD3 mutants to promote resistance to PLX4720-induced apoptosis. All 3 mutant forms of FOXD3 were able to protect against PLX4720-induced apoptosis similar to wild-type FOXD3. Thus, as outlined in the proposal, we are performing a systematic domain deletion analysis to more broadly define regions of interest.

iii) Knockdown FOXD3 in melanoma cell lines and determine effects on formation of melanoma spheres, self-renewal, and regeneration.

These studies have not been initiated yet.

iv) Knockdown FOXD3 in fresh melanoma tumors and determine effects on formation of melanoma spheres, self-renewal, and regeneration.

These studies have not been initiated yet.

Task 3. To test for mechanisms regulating FOXD3 in mutant B-RAF melanomas

i) Perform 'erase and replace' experiments to determine the ERK2 effector domains required for FOXD3 repression.

FOXD3 induction is mediated by depletion of ERK2 but the downstream effectors are unknown. The RAF-MEK-ERK cascade bifurcates at ERK with activation of kinases [6], and induction of intermediate early genes [7]. In this task, we proposed to test docking domain mutants that distinguish subsets of ERK2 effectors in the regulation of FOXD3 expression. Two main effector binding sites are present in ERK2, the Y261 regulates effectors containing DEF domain sites and controls immediate early gene expression [8, 9] and secondly the common docking domain around D319 interacts with motifs in RSKs, MSK and MNK [10, 11]. We have generated lentiviruses to express wild-type ERK2, ERK2-Y261A (which doesn't bind DEF domain containing proteins), and ERK2-D319A (which doesn't bind RSK, MSK and MNK). These mutants retain normal activation and nuclear translocation [8, 9]. We are currently performing erase-and replace experiments to knockdown endogenous ERK2 and re-express wild-type and mutant forms of ERK2 in mutant B-RAF melanoma cells. We plan to measure the ability of wild-type and mutant ERK2 to down-regulate FOXD3 expression by Western blotting.

ii) Express constitutively active forms of PI3K and Akt3 and determine effects on adhesion regulation of FOXD3 expression.

Our preliminary data showed that up-regulation of FOXD3 following B-RAF-inhibition is dependent on adhesion to fibronectin and also that inhibition of PI-3 kinase impairs up-regulation of FOXD3 levels. Thus, we proposed to determine whether activation of the PI-3 kinase/Akt pathway is sufficient to promote FOXD3 expression in non-adherent conditions. To this end, we expressed activated forms of PI-3 kinase (PI3K-CAAX) or Akt3 (myristylated Akt3) in WM793 cells and determined their ability to promote expression of FOXD3 in non-adherent cells treated with PLX4720. Our results showed that neither PI3K-CAAX nor myristylated Akt3 promoted FOXD3 up-regulation following B-RAF inhibition. As an alternative approach, we are currently analyzing the role of the axin/beta-catenin pathway in the regulation of FOXD3 following B-RAF inhibition.

## Key Research Accomplishments

1. Established systems to analyze the response of melanoma xenografts to RAF inhibitors in vivo.
2. Mutational analysis of FOXD3 and ruled out role of serine 46 phosphorylation and Groucho-binding domain in resistance to PLX4720-induced apoptosis.

## Reportable Outcomes

None to date

## Conclusion

In the first year of this grant, we have made substantial advances towards our desired goal of determining the role of FOXD3 in resistance to PLX4032/4720, defining the role of FOXD3 in melanoma cell protection from apoptosis and stem cell-like properties, and identifying mechanisms regulating FOXD3 in mutant B-RAF melanomas.

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## Appendices

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